



ELSEVIER

Journal of Chromatography A, 857 (1999) 263–273

JOURNAL OF
CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Studies on the relationship between structure and electrophoretic mobility of α -helical and β -sheet peptides using capillary zone electrophoresis[☆]

Balvant R. Sitaram, Hooi H. Keah, Milton T.W. Hearn*

Centre for Bioprocess Technology, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia

Received 25 December 1998; received in revised form 23 June 1999; accepted 24 June 1999

Abstract

The electrophoretic behaviour of a series of 33 different synthetic peptides has been investigated using free solution high-performance capillary zonal electrophoretic (HPCZE) methods. The dependency of the electrophoretic mobility, μ_{em} , on the peptide charge, q , and on the charge-to-size ratio parameter, ζ , determined according to several electromobility models, have been examined. Significant divergences from linearity in the μ_{em} vs. q or the μ_{em} vs. ζ plots were noted for several peptides, possibly due to the proclivity of specific arrangements of their amino acid sequences to assume preferred α -helical or β -sheet conformational features rather than random coil structures under the HPCZE conditions. These results provide further demonstration of the facility of HPCZE procedures to probe the effects of compositional, sequential and conformational differences of closely-related peptides and their consequences on their physicochemical behaviour in solution. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Structure–mobility relationships; Peptides

1. Introduction

Over the past decade, the application of high-performance capillary electrophoresis (HPCE) in its various selectivity modes has become a very valuable adjunct to high-performance liquid chromatography (HPLC) for the analysis of biomacromolecules [1,2]. For synthetic peptides, in particular, both

HPLC and HPCE now form essential components of the analytical characterisation of these molecules [3]. Increasingly, zonal, micellar or (biospecific) affinity-based HPCE procedures with open capillary systems are being adapted to allow resolution with extremely high separation efficiencies (e.g. $>10^5$ plates/m) of synthetic or naturally-occurring peptides as part of the determination of their structural, biophysical or functional properties. Since HPCE separations can be achieved with picomole to attomole quantities of peptidic analytes at nl/min flow-rates using relatively simple aqueous or aquo–organic solvent buffer systems [4–6], these methods represent very economical approaches to acquire essential information on the molecular properties of peptides under various

[☆]HPLC of Amino Acids, Peptides and Proteins, Part CXCIV. For Part CXCI see Ref. [37].

*Corresponding author. Tel.: +61-3-9905-3720; fax: +61-3-9905-5882.

E-mail address: milton.hearn@med.monash.edu.au (M.T.W. Hearn)

bulk solution and ligand-interaction conditions. Moreover, recent successes in interfacing high-performance capillary zonal electrophoresis (HPCZE) with electrospray mass spectrometry (ES-MS) and single cell biosensors has potentially provided an ideal system to permit studies of the structure–activity relationships of biologically active peptides under conditions that allow a very high level of discrimination of their compositional and functional integrity [7–11]. When operated under these zonal modes, or alternatively under affinity interaction modes with buffers containing lipophilic, metal ion-chelated ligands or bioselective ligands, at low and/or high temperatures, the opportunity also exists to explore the conformational properties that these peptides can manifest in different solution milieus [12,13]. Recent work in this laboratory has addressed several of these issues. Our aim has been to examine the relationships between electrophoretic mobility and the primary and/or secondary structures of peptides and peptidomimetics of interest as drug candidates in structure–activity studies related to peptide-based vaccines [14,15], cystine knot glycoprotein and growth factor antagonists [16–18], sperm tail and other gonadal proteins [19,20] and hypoglycaemic agonists [21] exploiting inter alia this interface between HPCZE and molecular characterisation.

As part of this work, we have recently synthesised and determined the electrophoretic mobilities (μ_{em} values) of a range of peptides that involve α -helical, linear β -strand or alternatively β -sheet/type II' β -turn loop structures related to (i) the coat protein of the human immuno-deficiency virus, HIV-1 gp120; (ii) various peptides related of the microtubule-associated protein-2 (MAP-2); and (iii) peptides related to the cysteine-rich secretory protein (CRISP) family of sperm tail proteins. Previously, no systematic work has been described in the scientific literature on the relationship between the structural features of these α -helical or β -sheet peptides and their μ_{em} properties. The availability of this set of 33 peptides thus has provided an opportunity to examine their electrophoretic behaviour in terms of physico-chemical relationships that link μ_{em} with the effective charge, q , the molecular mass, M_r , and the frictional size to mass ratio parameter, ζ . In addition, this study has permitted further evaluation of the contri-

bution that differences in primary and secondary structures, e.g. α -helical, β -sheet or random coil features, can make to electrophoretic mobility of peptides in these HPCZE systems.

2. Experimental

2.1. Materials

Trifluoroacetic acid (TFA), *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluoro-phosphate (HBTU), 1-hydroxybenzotriazole (HOBT), *N,N*-dimethylformamide (DMF), piperidine, 4-methylbenzhydramine resin (MBHA) hydrochloride (0.83 mmol/g), Wang resin with the first amino acid attached, and L- α -(9-fluorenylmethoxycarbonyl) (Fmoc)-protected amino acids were obtained from Auspep (Melbourne, Australia). Thioanisole, ethanedithiol, acetic anhydride, diisopropylethylamine (DIEA) and trifluoromethanesulfonic acid (TFMSA) were obtained from Aldrich (Milwaukee, WI, USA). Diisopropylcarbodiimide was obtained from Sigma (St. Louis, MO, USA). Fmoc-Glu (Rink amide mBHA)-OtBu resin (0.34 mmol/g), obtained from Novabiochem (Melbourne, Australia), was used for the synthesis of peptide **14**, whilst the C-terminal amide peptides were synthesised on the 4-methylbenzhydramine resin. Phenol was obtained from Merck Australia (Kilsyth, Australia). All solvents were of analytical grade or LC standard.

2.2. Instrumentation

Capillary electrophoretic separations were performed using a Beckman P/ACE 5000 Series analytical capillary electrophoresis system fitted with a diode array detector. The system was interfaced with a Pentium Computer utilising the System Gold software (version 7.1) for instrument control and data collection (Beckman, Fullerton, CA, USA). Preparative reversed-phase high-performance liquid chromatography (RP-HPLC) of the synthetic peptides was performed using a chromatographic system consisting of two Waters Model 600 solvent delivery pumps and an automated gradient controller. All samples were introduced into the column by means of a Rheodyne 7125 injector fitted with a 1 ml loop.

Detection was carried out using Model 441 UV absorbance detector. Analytical separations of the crude and purified peptide samples were conducted using a HPLC system consisting of a WISP Model 701B autosampler, an automated gradient controller and Model 486 variable-wavelength UV detector. The systems were interfaced to a Pentium Computer utilising the Waters Millennium software (version 2.1) for instrument control and data collection (Waters Assoc. Milford, MA, USA). Electrospray mass spectroscopy was carried out on a Perkin Elmer-Sciex Mass Spectrometer model “PE Sciex API//”.

2.3. Peptide synthesis

2.3.1. Synthesis and cleavage from the resin

Solid-phase peptide synthesis was performed either manually or using a PS3 Protein Technologies Automated Peptide Synthesiser, Rainin (Woburn MA, USA). Peptides were synthesised with standard Fmoc synthesis protocols with double coupling carried out where necessary as described previously [22]. The procedure involved the use of an appropriate amount of Fmoc amino acids (2 equiv.), HOBt (2 equiv.) and HBTU (2 equiv.) dissolved in DMF (6 ml) with 6 drops of DIEA added. Coupling reactions were allowed to proceed for 1 h. For deprotection, the Fmoc-protected resin was treated twice with 20% (v/v) aqueous piperidine in DMF for 5 min.

2.3.2. TFA cleavage

The ice-cooled peptide resin (0.5–1.0 g) was placed in a round bottom flask containing a magnetic stirrer and treated with an ice-cooled mixture of phenol (0.75 g), thioanisole (0.5 ml), ethanedithiol (0.25 ml), distilled water (0.5 ml) and TFA (10 ml). The flask was stoppered and stirred at room temperature for 2–3 h depending on the amino acid in the sequence. Upon completion of the cleavage reaction, the ‘peptide+resin’ mixture was filtered through a glass sinter and the flask rinsed with 2–3 ml of TFA. The solution was concentrated in vacuo and treated with cold ether to precipitate the peptide. The ether solution was kept at –20°C overnight before being filtered. The residue was taken up in water–acetonitrile (50:50, v/v) and lyophilised.

2.3.3. TFMSA cleavage

Peptide amides were synthesised using the MBHA resin and cleaved with a TFA – followed by a TFMSA protocol [14,22]. The “peptide+resin” mixture from the TFA cleavage was filtered and the resin washed with TFA (2×2 ml). The resin was then transferred to a 50 ml round bottom flask containing a magnetic stirrer. The resin was chilled, thioanisole (1 ml) and ethanedithiol (0.5 ml) added, stirred for 10 min, followed by addition of TFA (10 ml) with stirring for a further 10 min. The TFMSA (1 ml) was added dropwise with stirring. The flask was stoppered and stirred at 20°C for 2 h. Ice-cooled diethyl ether (50 ml) was added and the mixture was stirred vigorously for 1 min and then filtered. The peptide was extracted into TFA (3×3 ml), the solution concentrated in vacuo and then treated with cold ether to precipitate the peptide. The ether solution was kept in the refrigerator overnight before being filtered. The product was taken up in water–acetonitrile (50:50, v/v) and lyophilised.

2.4. Peptide purification and analysis

Purification of crude peptides was performed by RP-HPLC using a TSK-ODS-120T C₁₈ column (300×21.5 mm I.D., particle size 10 μm) protected by a guard column. About 50 mg of crude peptide (dissolved in 1 ml of Buffer A or Buffer B depending on the solubility characteristics of the peptide) was injected onto the column. Separations were achieved using a linear gradient of 0–100% buffer B formed over 1 h from Buffer A, 0.1% TFA in water; and Buffer B, 0.1% TFA in water–acetonitrile (40:60 v/v) at a flow-rate of 6 ml/min with detection at 254 nm. Fractions (2 ml) were collected using a Pharmacia (Frac-100) fraction collector. Analytical comparisons of the crude and purified peptide samples were carried out on a TSK C₁₈ column (150×4.6 mm, particle size 5 μm). Samples were eluted using a 25 min linear gradient of 0–85% buffer B at a flow-rate of 1 ml/min with detection at 214 nm.

2.5. Mass spectroscopic analyses

Electrospray mass spectroscopy was carried out with a scan range was between 100–2000 u. The samples (30 μl, 1 mg/ml) were injected via a ‘PE

ISS 200' auto injector. The solvent used was water–acetonitrile (40:60, v/v), 0.1% acetic acid.

2.6. Capillary electrophoresis

Phosphate buffers were prepared by titrating 50 mM sodium dihydrogenphosphate with phosphoric acid to pH 3.0. Prior to analysis, samples of the synthetic peptides were placed in 1 ml Eppendorf tubes and diluted with 0.1 M phosphoric acid to a concentration of 1 mg/ml. The diluted samples were centrifuged at 11 000 rpm for 10 min in a micro-centrifuge (Biofuge B, Heraeus Christ, Osterode, Germany) to remove any particulate materials. The supernatant as aliquots (200 μ l) was transferred to 1 ml Eppendorf tubes and stored at 5°C or at –20°C until required for analysis.

Separation of the synthetic peptides using capillary electrophoresis was achieved using eCAP plain fused-silica capillaries (Beckman) without coating. The capillaries were 50 μ m I.D. and 375 μ m O.D., and were of a total length of 47 cm, with length of 40 cm to the detector. For analytical separation the following method was routinely used with these capillaries: (i) a 2 min rinse with separation buffer; (ii) 7 s pressure injection of a peptide solution 1 mg/ml in 0.1 M phosphoric acid, followed by a 4 s pressure injection of neutral marker DMF, (0.02% w/w in water); (iii) separation at 15 kV (constant voltage with the inlet as the anode and the outlet as cathode); and (iv) a 3 min wash with 0.1 M NaOH, a 3 min wash with water, a 3 min wash with 0.1 M HCl, a 3 min wash with water and a 8 min wash with separation buffer. All electrophoretic separations were carried out at least in duplicate at constant voltage (typically 15 kV) and at a capillary temperature of 25°C. Detection was performed by UV absorbance spectroscopy at 200 nm.

2.7. Calculation of the electrophoretic mobilities and charge of the synthetic peptides

The effective electrophoretic mobilities, μ_{em} s, of the different peptides were determined following HPCE on a 40 cm capillary using in 50 mM sodium phosphate buffer pH 3 at 25°C, with an applied potential of 15 kV. Electroosmotic flow was estimated by determining the migration time of DMF, a

neutral marker included in all analyses. The charge, q , associated with each peptide sequence, assuming an average random coil conformation for the species under the pH conditions of the separation, was calculated using the Henderson–Hasselbach equation [23]. The charge, q (or notional valence), was defined as the sum of the fractional charges carried by each amino acid residue in the sequence at pH 3.0. The pK_a values for the N-terminal, C-terminal and side-chain functional groups of the individual amino acid residues incorporated in the peptide sequences studied were obtained from Rickard et al. [24]. Calculations based on the pK_a values for the same functional groups in the free amino acids were also utilised. The pK_a values for the free amino acids were obtained from Dawson et al. [25].

3. Results and discussion

The amino acid sequences, ratios of migration time of peptide/migration time of DMF as the neutral marker, molecular masses (M_r values), calculated effective charges (q values), effective electrophoretic mobilities (μ_{em} values) and calculated frictional drag parameters, (ζ values) of the synthetic α -helical, β -sheet and random coil peptides **1–33**, studied with the HPCZE system described in the Experimental section, are presented in Table 1. It can be noted that the peptides **1–13** represent overlapping fragments of the parent peptide **14**, whereby the N- or C-terminal regions have been progressively truncated. In addition, peptides **15–17** represent either the C-terminal amide variants of **14** or alternatively Asp \rightarrow Leu substitutions of peptide **14**. These peptides **1–17** form part of a set of highly immunogenic peptides, which elicit antibodies that cross-react with the HIV-1 gp120 coat protein in biochemical and immunological assays. With the higher-molecular-mass members of this peptide set, i.e. with peptides **14** or **15**, etc., solution conformational measurements based on COESY and NOESY two-dimensional NMR and CD spectroscopy at low pH conditions, i.e. pH 2.3, have indicated [14,15,26] a considerable α -helical presence extending over the region from Asp⁶ to Ala¹³ of these peptides, with the amide exchange rates consistent with H bond formation expected for this type of secondary structure.

Table 1
Sequence, physicochemical and electrophoretic parameters for the different synthetic peptides **1–33**

| Peptide code | Sequence | Ratio migration time sample/marker | M_r (Theoretical) | M_r (Actual) ^a | Calculated effective charge, q^b | Effective electrophoretic mobility, μ_{em} ($\text{cm}^2/\text{V s} \times 10^5$) | Effective frictional drag parameter ($\zeta = q/M_r^{2/3} \times 10^2$) |
|--------------|--|------------------------------------|---------------------|-----------------------------|------------------------------------|---|---|
| 1 | KNWDRAPQ-COOH | 0.142 | 1014.0 | 1014 | 2.37 | 17.66 | 2.35 |
| 2 | DDKNWDRAPQ-COOH | 0.195 | 1244.2 | 1244 | 1.89 | 11.64 | 1.64 |
| 3 | LYDDKNWDRAPQ-COOH | 0.183 | 1520.6 | 1519 | 1.89 | 13.04 | 1.43 |
| 4 | DDALYDDKNWDRAPQ -COOH | 0.316 | 1821.9 | 1822 | 1.41 | 7.19 | 0.95 |
| 5 | APQRCYYQ-COOH | 0.166 | 1100.1 | 1099 | 1.61 | 13.46 | 1.51 |
| 6 | DRAPQRCYYQ-COOH | 0.155 | 1371.4 | 1372 | 2.37 | 14.46 | 1.92 |
| 7 | NWDRAPQRCYYQ-COOH | 0.154 | 1671.7 | 1672 | 2.37 | 14.75 | 1.69 |
| 8 | DDKNWDRAPQRCYYQ-COOH | 0.178 | 2030.1 | 2028 | 2.89 | 13.97 | 1.80 |
| 9 | DRAPQRCY-COOH | 0.191 | 1080.2 | 1079 | 2.37 | 13.95 | 2.22 |
| 10 | DRAPQRAY-COOH | 0.144 | 976.0 | 976 | 2.37 | 15.95 | 2.41 |
| 11 | DAPQRCYYQ-COOH | 0.272 | 1215.2 | 1214 | 1.37 | 8.55 | 1.21 |
| 12 | RAPQRCYYQ-COOH | 0.160 | 1256.3 | 1255 | 2.61 | 16.81 | 2.24 |
| 13 | DAAPQRCYYQ-COOH | 0.273 | 1286.3 | 1287 | 1.37 | 8.81 | 1.16 |
| 14 | DDALYDDKNWDRAPQRCYYQ-COOH | 0.209 | 2607.8 | 2607 | 2.41 | 11.69 | 1.27 |
| 15 | DDALYDDKNWDRAPQRCYYQ-CONH ₂ | 0.175 | 2606.8 | 2606 | 1.80 | 15.24 | 0.95 |
| 16 | DLALYDDKNWDRAPQRCYYQ-CONH ₂ | 0.174 | 2605.9 | 2604 | 2.04 | 15.36 | 1.08 |
| 17 | DDALYLDKNWDRAPQRCYYQ-CONH ₂ | 0.165 | 2605.9 | 2603 | 2.04 | 15.54 | 1.08 |
| 18 | CKRIHIGPGRAFYTTC-COOH | 0.125 | 1967.8 | 1964 | 4.36 | 21.01 | 2.78 |
| 19 | KQYYWYAWCQPPQDLIMD-COOH | 0.315 | 2676.6 | 2675 | 0.88 | 7.49 | 0.46 |
| 20 | LKRDQEPMDFHWDYDLKRD-COOH | 0.148 | 2621.1 | 2620 | 4.13 | 19.65 | 2.17 |
| 21 | DFHIWDDYLRDQEPMDFHI-COOH | 0.172 | 2621.1 | 2620 | 3.38 | 16.18 | 1.78 |
| 22 | KDLRWHDIRWHDNRQ-COOH | 0.113 | 2783.3 | 2783 | 7.65 | 25.52 | 3.87 |
| 23 | RNDHWRIDKWRIDKWDLDKY-COOH | 0.119 | 2759.4 | 2759 | 6.41 | 24.26 | 3.28 |
| 24 | TPPTDWLADRHEMDQNKDDK-COOH | 0.152 | 2412.6 | 2413 | 3.38 | 18.42 | 1.88 |
| 25 | VHNQLVLRRLAPSPVVLFF-COOH | 0.176 | 2270.8 | 2271 | 3.61 | 15.47 | 2.09 |
| 26 | QLQYQREIVNKHNLNLR-COOH | 0.147 | 2003.1 | 2004 | 4.55 | 19.07 | 2.86 |
| 27 | TPYQQGTPCACOOH | 0.278 | 1137.1 | 1137 | 0.61 | 7.46 | 0.56 |
| 28 | AGCEHELLKECKAT | 0.122 | 1804.5 | 1803 | 4.52 | 20.1 | 3.05 |
| 29 | CALEEADDDRS-COOH | 0.209 | 1295.5 | 1295 | 0.83 | 11.44 | 0.68 |
| 30 | CKTGRGISTPERKVAK-COOH | 0.117 | 1959.6 | 1960 | 6.58 | 24.18 | 4.20 |
| 31 | CGPEKGKAAK-COOH | 0.111 | 1060.5 | 1060 | 3.58 | 24.90 | 3.44 |
| 32 | CASSLRNDSS-COOH | 0.187 | 1111.4 | 1110 | 1.37 | 13.50 | 1.28 |
| 33 | CFMLPPVAASSLRNDSS-COOH | 0.242 | 1867.4 | 1867 | 1.37 | 9.750 | 0.91 |

^a Determined by ESI-MS.

^b Calculated using pK_a values for amino acids within peptide structures as reported by Rickard et al. [24].

The carboxyl- and amino- terminal regions of these larger peptides on the other hand appear to have only extended/random structures in solution. Moreover, as the sequence region flanking the N-terminal side of the amino acid array of ...D⁶KNWDRA¹³... becomes shorter, the extent of preferred secondary structure is also reduced. Hence, peptide **4** is more similar to peptides **14** or **15** in terms of preferential stabilisation of the secondary structure than, for example, peptides **2** or **8**. As the proline residue, i.e. Pro¹⁴ in peptide **14**, introduces a

partial β -turn kink in the secondary structure of these peptides, this results in termination of the preceding α -helical features of the secondary structure. The smaller members of this set, containing only short segments derived from the N-terminal, C-terminal or internal sequences of the parent peptide, e.g. peptides **5**, **9**, etc., show greatly reduced or little propensity to adopt a preferred secondary structure under similar low pH conditions. In contrast, peptide **18** represents a fragment of the V3-loop region of gp120, and also shows little secondary structural preferences as the

linear peptide, but can adopt antiparallel β -strand loop features when constrained as the disulphide bonded cyclic peptide.

Preliminary comparisons of the derived q values of the individual fragment peptides **1–13** with their effective electromobility values, μ_{em} , suggested that a relationship may exist between the overall charge, q , and μ_{em} . If the incremental contributions of the fractional charges of the individual amino acid residues with a peptide were the sole determinant of the μ_{em} , then linear additivity of μ_{em} on q for this peptide set would be anticipated. However, linear regression analysis of these experimental data [which took the form $\mu_{em} = (5.27 \cdot 10^{-5}) q + (2.19 \cdot 10^{-5})$] yielded a correlation coefficient of $r^2 = 0.679$. On incorporating the corresponding results for the larger peptides, **14–18**, the linear regression analysis [which took the form $\mu_{em} = (3.82 \cdot 10^{-5}) q + (5.43 \cdot 10^{-5})$] resulted in a slightly increased correlation coefficient of $r^2 = 0.686$. The prevalence for outlying data values to occur with various members of this structurally similar set of peptides thus confirmed that factors other than the summated fractional charge, q , calculated according to the Henderson–Hasselbach equation [23], were influencing the μ_{em} values of some members of this peptide set. In addition to q , the contribution of molecular frictional drag and the influence of the array arrangement of the amino acid residues within a particular sequence can be identified as two significant factors that potentially could affect the electromobility of peptidic or protein substances in HPCZE systems. A number of mathematical models have been developed to describe the combined effects on μ_{em} of the charge and the molecular frictional drag of macromolecules in HPCZE systems. According to Cifuentes and Poppe [27,28], an optimum fit of the dependency that links μ_{em} to the respective charge-size parameters is likely to be observed when μ_{em} is plotted against ζ , where $\zeta = q/M_r^{2/3}$. Although this relationship has been widely used, other investigators [3,24,29,30] have proposed that plots of μ_{em} vs. $q/M_r^{1/3}$, μ_{em} vs. $q/M_r^{1/2}$ or μ_{em} vs. $\ln(q+1)/N^{0.43}$, where N is the number of amino acid residues in the sequence, can also be employed. However, several reports using these alternative approaches have suggested that they have lower predictive fidelities.

When μ_{em} was plotted against ζ ($=q/M_r^{2/3}$) for

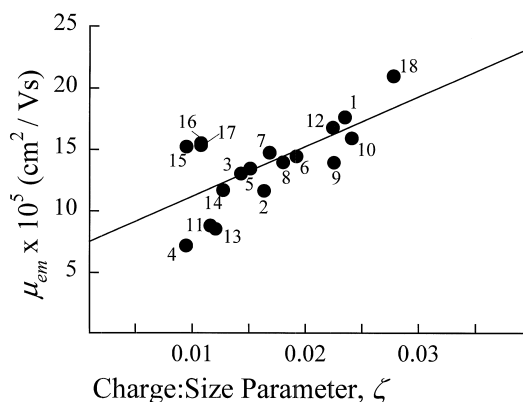


Fig. 1. Fit of μ_{em} vs. ζ (calculated according to the relationship $\zeta = q/M_r^{2/3}$) for the peptides **1–18** separated using a 50 mM sodium phosphate buffer pH 3.0. Other conditions are given in the Experimental section. The charge, q , was calculated [24] using pK_a values for amino acid side chains within peptides.

this set of **1–18** HIV-1 peptide mimetics, linear regression analysis (Fig. 1) took the form $\mu_{em} = (4.06 \cdot 10^{-3})\zeta + (7.11 \cdot 10^{-5})$ with a correlation coefficient of $r^2 = 0.461$. In contrast, when the initial set of fragment peptides **1–13** was employed, the plot of μ_{em} vs. ζ took the form $\mu_{em} = (6.06 \cdot 10^{-3})\zeta + (2.57 \cdot 10^{-5})$ with a correlation coefficient of $r^2 = 0.819$, representing a significant improvement in correlation for a linear dependency of μ_{em} on ζ . Closer inspection of the HPCZE experimental data for peptides **1–18** indicated that in all cases where the experimentally derived μ_{em} and the calculated q and ζ values resulted in outlying data compared to the corresponding values predicted according to a linear dependency of μ_{em} on q , or μ_{em} on ζ , then these peptides have been found by CD or $^1\text{H-NMR}$ techniques [15,26] to be able to adopt under low pH conditions stable α -helical structures as a direct consequence of the particular amino acid array within their sequences. When this subgroup of conformationally more constrained peptides were removed from the analysis of the data for peptides **1–18**, then the remaining peptides, i.e. those peptides within this set that exist on average as random coil structures or are unable to assume a significant population of preferred secondary structure(s) under low pH conditions, showed an improved correlation for a linear dependency of μ_{em} on q or μ_{em} on ζ .

This conclusion was tested using other peptide mimics of the HIV-1 gp120 protein, namely the synthetic peptides **19–25**, which according to Chou-Fasman CD-based procedures have little preference for α -helical or β -sheet structures under low pH conditions. The amino acid sequences, ratios of migration time of peptide/migration time of the neutral marker DMF, molecular masses, the calculated effective charges, the effective electrophoretic mobilities and the calculated frictional drag parameters, for the same HPCZE system with peptides **19–25** are also presented in Table 1. In these cases, examination of the experimental data for q of the individual peptides in this group and their μ_{em} values indicated a linear relationship existed between q and μ_{em} , i.e. $\mu_{em} = (2.61 \cdot 10^{-5}) q + (7.19 \cdot 10^{-5})$ with a correlation coefficient of $r^2 = 0.914$. The narrow molecular mass range encompassed within this group of peptides **19–25** also enabled the contribution of the molecular mass dependencies, reflected in the molecular frictional drag term ζ , on the μ_{em} term to be more precisely ascertained. As is evident from Fig. 2, which illustrates the plot of μ_{em} vs. ζ [where $\mu_{em} = (5.27 \cdot 10^{-3}) \zeta + (6.41 \cdot 10^{-5})$ with a correlation coefficient of $r^2 = 0.928$], the results are consistent with the conclusion that the effect of differences in molecular mass per se on μ_{em} are minimal within this series of peptides and that the observed changes in μ_{em} for this group of peptides are predominantly

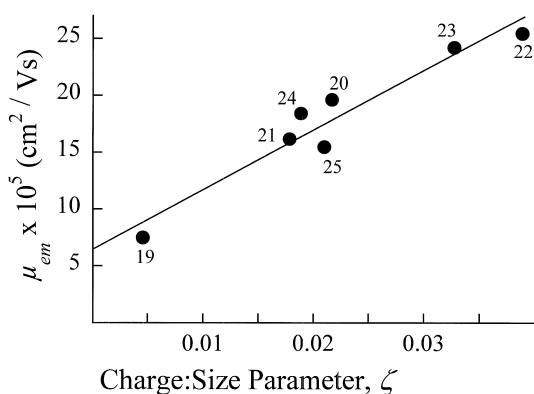


Fig. 2. Fit of μ_{em} vs. ζ (calculated according to the relationship $\zeta = q/M_r^{2/3}$) for the peptides **19–25** separated using 50 mM sodium phosphate buffer pH 3.0. Other conditions are given in the Experimental section. The charge, q , was calculated [24] using pK_a values for amino acid side chains within peptides.

due to the dependence of μ_{em} on q and thus on the relationship that links μ_{em} and the amino acid composition.

As a further example with peptides that can assume β -sheet structures in low pH buffer systems similar to those employed in these experiments, the HPCZE behaviour of synthetic peptides related to the microtubule-associated protein-2 (MAP-2) and the sperm tail protein, tpx-1, a member of the CRISP superfamily of cysteine rich proteins, were also investigated. The amino acid sequences, ratios of migration time of peptide/migration time of the neutral marker DMF, molecular masses, the calculated effective charges, the effective electrophoretic mobilities and the calculated frictional drag parameters, for these peptides, **26–33**, are presented in Table 1. Examination of the relationship between the q -value of individual peptides in this series and their μ_{em} values indicated a lower correlation ($r^2 = 0.778$) for a linear relationship [$\mu_{em} = (2.68 \cdot 10^{-5}) q + (8.44 \cdot 10^{-5})$] between μ_{em} and q . Again, the presence of a number of outlying experimental values indicated that factors other than q (calculated according to the assumption that the peptides assumed random coil conformations) were influencing the μ_{em} values of these peptides. Fig. 3 illustrates the plot of μ_{em} vs. ζ [$\zeta = q/M_r^{2/3}$] for the tpx-1 peptides together with the superimposed linear regression line [$\mu_{em} = (4.57 \cdot 10^{-3}) \zeta + (6.64 \cdot 10^{-5})$] where the derived correlation

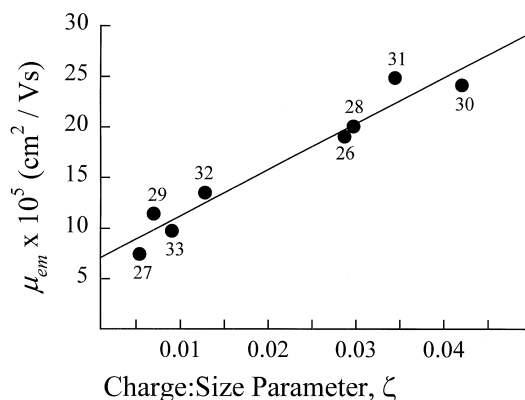


Fig. 3. Fit of μ_{em} vs. ζ (calculated according to the relationship $\zeta = q/M_r^{2/3}$) for the peptides **26–33** separated using 50 mM sodium phosphate buffer pH 3.0. Other conditions are given in the Experimental section. The charge, q , was calculated [24] using pK_a values for amino acid side chains within peptides.

coefficient assuming a linear relationship between μ_{em} and ζ was $r^2=0.946$.

It is evident from the above results with the peptides **1–33** that improved correlations could be obtained between the experimentally-derived values of μ_{em} and the derived values of q or ζ [$=q/(M_r)^{2/3}$] calculated using the Henderson-Hasselbach equation, only when the peptide is assumed or known to behave on average as random coil conformational species under the pH conditions of the HPCZE separation. Moreover, within a set of peptides of similar size and similar apparent conformational features, improved correlations were also observed (cf. data for the peptides **19–25**). These findings would account for the differences between the predicted and observed electromigration behaviour of peptides previously measured [24,31] under various HPCZE conditions. As evident from the plots for the various peptides investigated in the present study, the simple introduction of a term to account for the molecular frictional drag per se, based on the empirical approaches of Rickard et al. [24] or Himmel-Squire [32], which assume that the peptide adopts on average a globular shape in solution that corresponds to the dimensions of the random coil structure, does not lead to a marked enhancement in the degree of correlation for peptides **1–33** with the studied low pH HPCZE system. This outcome was particularly apparent when the data for all peptides, **1–33**, were included as a single plot of μ_{em} vs. q with the data calculated using $\text{p}K_a$ values for amino acids within peptides as described by Rickard et al. [24]. Linear regression analysis of these data [$\mu_{\text{em}}=(2.65 \cdot 10^{-5})q+(7.91 \cdot 10^{-5})$] yielded as expected a slightly improved correlation coefficient of $r^2=0.781$, due to the inclusion of an increased number of peptides with random coil structures. Various outlying examples were again evident, i.e., peptides **4, 15, 16, 31**, for those peptides with a high propensity to assume a preferred secondary structure under these low pH HPCZE conditions. Similarly, divergent electrophoretic behaviour was evident for this same group of 33 peptides when the combined data were plotted as μ_{em} vs. ζ [$=q/(M_r)^{2/3}$] (Fig. 4a). Linear regression analysis of these data [$\mu_{\text{em}}=(4.81 \cdot 10^{-3})\zeta+(6.28 \cdot 10^{-5})$] yielded a slightly higher correlation coefficient of $r^2=0.818$. In order to ascertain the extent of variation in r^2 values when alternative electromobili-

ty correlations were employed, the corresponding analyses were carried out using the empirical relationships $\zeta=q/M_r^{1/3}$, $\zeta=q/M_r^{1/2}$ and $\zeta=\ln(q+1)/N^{0.43}$ with the data for q again calculated using $\text{p}K_a$ values for amino acids within peptides as described by Rickard et al. [24]. In Fig. 4b–d are shown the plots of μ_{em} vs. ζ , utilising these alternative ζ dependencies. Comparison of the results shown in Fig. 4a–d revealed that the alternative relationships based on molecular mass, i.e. $\zeta=q/M_r^{1/3}$ or $\zeta=q/M_r^{1/2}$, provided similar overall trends in the corresponding plots of μ_{em} vs. ζ , for the majority of these peptides to that found with $\zeta=q/(M_r)^{2/3}$, with the dependency linking μ_{em} to the number of residues, N , within a particular sequence, showing the lowest correlation. Thus, the dependencies based on molecular mass provided correlation coefficients of $r^2=0.832$, $r^2=0.835$ and $r^2=0.838$, respectively, whilst for the dependency based on the number of residues within a specific sequence, a $r^2=0.733$ was obtained.

The question then arises whether the approximations inherent to the Henderson–Hasselbach equation, namely that the peptide is assumed to exist on average in a random coil conformation and that the overall charge, q , associated with each peptide sequence can be defined as the sum of the fractional charges carried by each amino acid residue at pH 3.0, lead to the reduced correlations evident for a linear relationship between μ_{em} and q or μ_{em} and ζ for the peptides investigated in the present study. Charge suppression effects can also contribute to the q value of a peptide, with over-estimated of the charge for peptides of high charge density one of the major contributors for the electromobility being over-estimated. Compensation for these effects can be made through the use [5,29] of the term $\ln(q+1)$ with the electrophoretic mobility then exhibiting a dependency on $(M_r)^s$, where the s -value can vary between 1/3 and 2/3. The s -values derived from the plots of $\log(M_r)$ vs. $\log[\ln(q+1)]/\mu_{\text{em}}$, however, did not permit a correlation to be established with the known solution properties of these peptides as determined by CD and two-dimensional-NMR spectroscopy, suggesting that other types of conformation effects may be operating in aqueous solutions compared to water–organic solvent systems commonly used with these spectroscopic methods. In terms of the Henderson–Hasselbach analysis a further po-

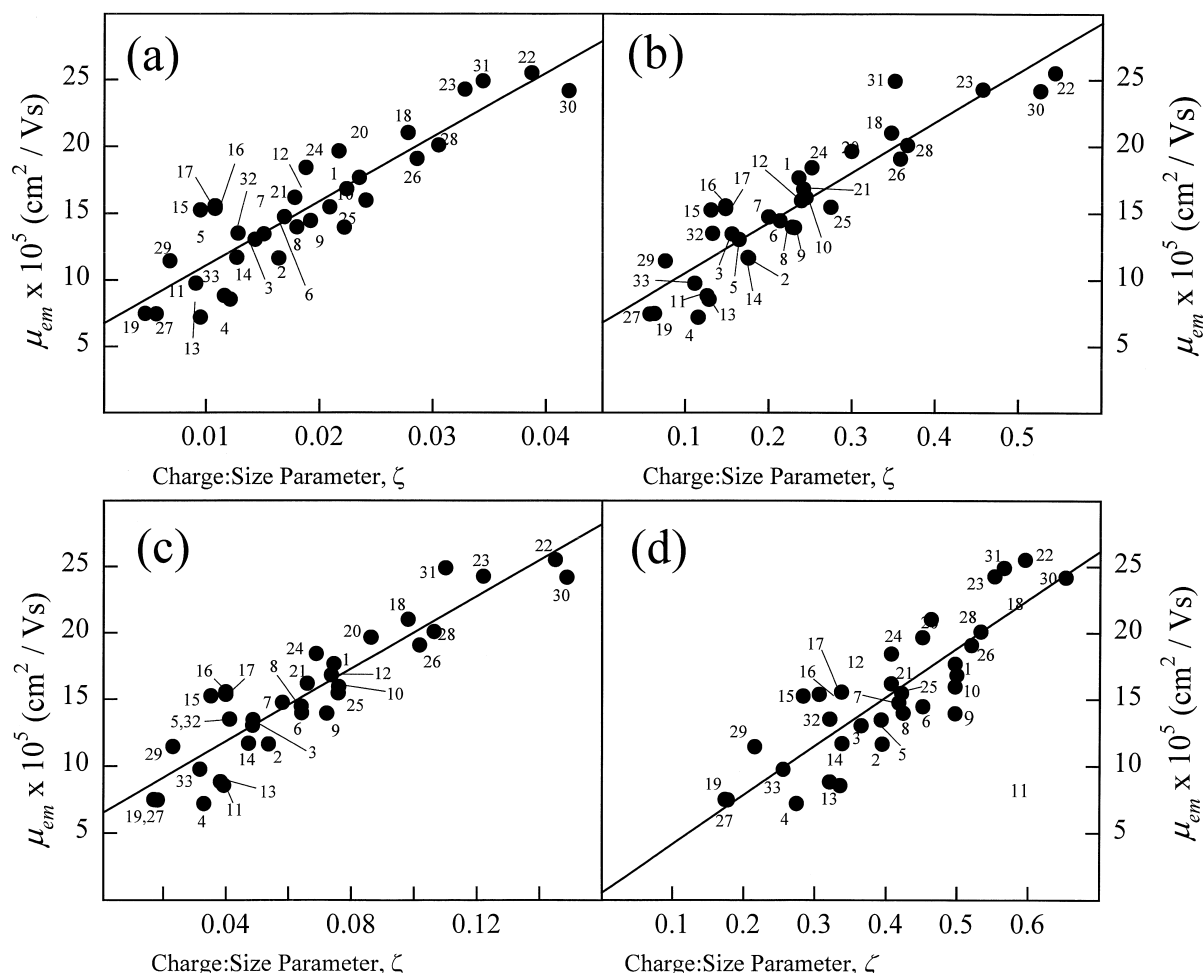


Fig. 4. (a) Fit of μ_{em} vs. ζ (calculated according to the relationship $\zeta = q/M_r^{2/3}$) for all peptides 1–33 separated using 50 mM sodium phosphate buffer pH 3.0. Other conditions are given in the Experimental section. In panels (b)–(d) are shown the plots of μ_{em} vs. ζ , calculated according to the relationship $\zeta = q/M_r^{1/3}$ and the alternative electromobility correlations of (b) $\zeta = q/M_r^{1/3}$, (c) $\zeta = q/M_r^{1/2}$ and (d) $\zeta = \ln(q+1)/N^{0.43}$, where N is the number of amino acid residues in the sequence. The charge, q , was calculated [24] using pK_a values for amino acid side chains within peptides.

tential influence exists, namely that the q values are calculated from pK_a values empirically derived [24] for amino acid side chains within a peptide sequence, rather than from the use of the corresponding pK_a values measured for free amino acids in solution.

The outcome of the use of these alternative pK_a values, for the peptides 1–33, indicated that use of pK_a values derived for free amino acids did not result in any significant improvement in the ensuing correlation coefficient for a linear dependencies of μ_{em} on ζ [i.e. where $\mu_{em} = (5.36 \cdot 10^{-3})\zeta + (5.99 \cdot$

$10^{-5})$; $r^2 = 0.865$], assuming that $\zeta = q/(M_r)^{2/3}$. By including only those peptides that can on average assume random coil structures under the low pH buffer conditions used in these studies, the correlation coefficient reached a value of $r^2 = 0.992$. As a consequence, these results clearly indicate that the propensity for a peptide to assume a preferred secondary structure as a direct consequence of the sequence array of its component amino acid residues has an important impact on the μ_{em} value and this conformational proclivity must be factored into the

interpretation of the electro-migrational properties of synthetic and naturally-occurring peptides in HPCZE investigations. Various co-solvent additives, such as 2,2,2-trifluoroethanol (TFE) or urea, can be employed to ascertain whether a polypeptide adopts a particular secondary structure in free solution. Several studies have recently been reported [2,27] on the use of such cosolvent additives in the HPCZE of peptides. Although changes in selectivity and effects on the Stokes radius of the polypeptide have been noted that are strongly dependent on the peptide sequence, the use of TFE and other cosolvent additives have been found [33,34] to significantly affect the electromobilities of even small tri- and tetrapeptides (e.g. GGNA), which are unable to assume any secondary structure. This finding is not surprising since the presence of such co-solvent additives will have a significant effect on the dielectric constant and viscosity of the electrophoretic buffer and these changes will profoundly influence the dissociation constants and Stokes radius characteristics of peptides in these HPCZE systems. In associated studies with the same polypeptides described in this paper, we have shown that in water-trifluoroethanol (60:40, v/v) solvent systems, very long electro-migration times were observed, associated with temperature instability, precluding reliable measurement of the relevant dissociation constants or Stokes radii of the larger polypeptides by HPCZE techniques under these water-organic solvent buffer conditions. Other workers have provided data consistent with this conclusion with polypeptides. Thus, the use of cosolvent conditions such as water-trifluoroethanol (60:40, v/v) did not permit additional insight to be gained into the nature of conformational status of the largest members of this test group of peptides. In addition, it can be noted that Rathore and Horváth [35] have demonstrated that *cis* and *trans* isomers of proline-containing peptides can be resolved by low temperature HPCZE methods, whilst topologically similar sets of L- α - and the retro-inverso D- α -peptide isomers containing proline residues have also been resolved [26,36] under analogous RP-HPLC and HPCZE experimental conditions. Collectively, these results indicate that HPCZE procedures provide a sensitive and rapid method to assess the compositional and conformational differences of related peptides in solution under different

buffer, pH and temperature conditions. Investigations addressing these possibilities will be reported in subsequent papers.

Acknowledgements

These investigations were supported by the National Health and Medical Research Council of Australia and the Australian Research Council.

References

- [1] B.L. Karger, A.S. Cohen, A. Guttman, J. Chromatogr. 492 (1989) 585–614.
- [2] I. Messana, D.V. Rossetti, L. Caaiano, F. Misiti, B. Giardina, M. Castagnola, J. Chromatogr. A 699 (1997) 149–171.
- [3] W. Voelter, J. Schutz, O.E. Tsitsiloni et al., J. Chromatogr. A 807 (1998) 135–149.
- [4] M. Harold, G.A. Ross, R. Grimm, D.N. Heiger, in: K.D. Altria (Ed.), Capillary Electrophoresis Guidebook: Principles, Operation and Applications, Humana Press, Totawa, NJ, 1996, pp. 258–308.
- [5] N.J. Adamson, E.C. Reynolds, J. Chromatogr. A 699 (1997) 133–147.
- [6] A.J. Smith, Meths Mol. Biol. 64 (1997) 91–99.
- [7] O. Orwar, K. Jardemark, I. Jacobson et al., Science 272 (1996) 1779–1782.
- [8] J.B. Shear, H.A. Fishman, N.L. Allbritton, D. Gangan, R.N. Zare, R.H. Scheller, Science 267 (1995) 74–77.
- [9] D.T. Chui, A. Hsiao, A. Gaggar, R.A. Garza-Lopez, O. Orwar, R.N. Zare, Anal. Chem. 69 (1997) 1801–1807.
- [10] H.A. Fishman, O. Orwar, R.H. Scheller, R.N. Zare, Proc. Natl. Acad. Sci. 92 (1995) 7877–7881.
- [11] K. Jardemark, O. Orwar, I. Jacobson, A. Mosche, R.N. Zare, Anal. Chem. 69 (1997) 3427–3434.
- [12] M.T.W. Hearn, in: M.A. Vijaylakshmi (Ed.), Theory and Practice of Biochromatography, Harwood Academic, Switzerland, 1999, in press.
- [13] K. Haupt, F. Roy, M.A. Vijaylakshmi, Anal. Biochem. 234 (1996) 149–154.
- [14] H.H. Keah, E. Kecorius, M.T.W. Hearn, J. Pept. Res. 51 (1998) 2–11.
- [15] K.A. Higgins, W. Bicknell, H.H. Keah, M.T.W. Hearn, J. Pept. Res. 50 (1997) 421–435.
- [16] P.T. Gomme, P.E. Thompson, J. Whisstock, P.G. Stanton, P.G., M.T.W. Hearn, J. Pept. Res., (1999) in press.
- [17] M.K. O'Bryan, K. Squire, M.P. Hedger, M.T.W. Hearn, D.M. DeKrester, J. Mol. Endocrinol., (1999) submitted for publication.
- [18] D.J. Phillips, J.R. McFarlane, D.M. DeKrester, M.T.W. Hearn, J. Endocrinol. 155 (1997) 65–71.

- [19] M.K. O'Bryan, K.A. Loveland, D. Herzfeld, J.F. McFarlane, M.T.W. Hearn, D.M. DeKretser, *Mol. Reprod. Dev.* 50 (1998) 313–322.
- [20] J.R. McFarlane, L.M. Foulds, A.E. O'Connor, D.J. Phillips, G. Jenkins, M.T.W. Hearn, D.M. DeKretser, *Endocrinology*, (1999) in press.
- [21] P.E. Thompson, N.J. Ede, N. Lim, F.M. Ng, I.D. Rae, M.T.W. Hearn, *Drug Design Discov.* 13 (1995) 55–72.
- [22] V. Cavallaro, P.E. Thompson, M.T.W. Hearn, *J. Pept. Sci.* 4 (1998) 335–343.
- [23] B. Skoog, A. Wickman, *Trend Anal. Chem.* 5 (1986) 82–83.
- [24] E.C. Rickard, M.M. Strohl, R.G. Neilson, *Anal. Biochem.* 197 (1991) 197–209.
- [25] R.M.C. Dawson, D.C. Elliot, W.H. Elliot, K.M. Jones, in: *Data for Biochemical Research*, 3rd ed, Oxford Science Publications, 1986, pp. 2–31.
- [26] R.I. Boysen, Y. Wang, H.H. Keah, M.T.W. Hearn, *J. Biophys. Chem.* 77 (1999) 79–97.
- [27] A. Cifuentes, H. Poppe, *J. Chromatogr. A* 680 (1994) 321–340.
- [28] A. Cifuentes, H. Poppe, *Electrophoresis* 18 (1997) 2362–2376.
- [29] P.D. Grossman, J.C. Colburn, H.H. Lauer, *Anal. Biochem.* 179 (1989) 28–33.
- [30] V.J. Hilser, G.D. Worisila, S.E. Rudnick, *J. Chromatogr.* 630 (1993) 329–336.
- [31] V. Kasicka, Z. Prusik, O. Smekal et al., *J. Chromatogr. B* 656 (1994) 99–106.
- [32] M.E. Himmel, P.G. Squire, *Int. J. Pept. Prot. Res.* 17 (1981) 365–373.
- [33] M. Castagnola, D.V. Rossetti, F. Misiti, L. Cassiano, B. Giardina, I. Messina, *J. Chromatogr. A* 792 (1997) 57–65.
- [34] M. Castagnola, L. Cassiano, I. Messina, M. Paci, D.V. Rossetti, B. Giardina, *J. Chromatogr. A* 735 (1996) 271–281.
- [35] A.S. Rathore, Cs. Horváth, *Anal. Biochem.* 70 (1998) 3069–3077.
- [36] M.T.W. Hearn H.H. Keah, R.I. Boysen, L. Cassiano, I. Messina, D. Rossetti, M. Castagnola, *Anal. Chem.*, (1999) in press.
- [37] M.T.W. Hearn, L.G. Zhao, *Anal. Chem.*, (1999) in press.